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Urease Characteristics and Phylogenetic Status of *Bacillus paralicheniformis*

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In 2015, *Bacillus paralicheniformis* was separated from *B. licheniformis* on the basis of phylogenomic and phylogenetic studies, and urease activity was reported as a phenotypic property that differentiates between the two species. Subsequently, we have found that the urease activity of *B. paralicheniformis* is strain-specific, and does not reliably discriminate between species, as strains having the same urease gene cluster were identified in *B. licheniformis* and *B. sonorensis*, the closest relatives of *B. paralicheniformis*. We developed a multilocus sequence typing scheme using eight housekeeping genes, *adk*, *ccpA*, *glpF*, *gmk*, *ilvD*, *pur*, *spo0A*, and *tpi* to clearly identify *B. paralicheniformis* from closely related *Bacillus* species and to find a molecular marker for the rapid identification of *B. paralicheniformis*. The scheme differentiated 33 *B. paralicheniformis* strains from 90 strains formerly identified as *B. licheniformis*. Among the eight housekeeping genes, *spo0A* possesses appropriate polymorphic sites for the design of a *B. paralichenofomis*-specific PCR primer set. The primer set designed in this study perfectly separated *B. paralicheniformis* from *B. licheniformis* and *B. sonorensis*.

Keywords: *Bacillus paralicheniformis*, *Bacillus licheniformis*, *Bacillus sonorensis*, urease, MLST, *spo0A*

Introduction

The genus *Bacillus* is the most populous bacterial group in soybean-based fermented foods of Asia. *Bacillus licheniformis* is a dominant *Bacillus* species identified from doenjang, a Korean fermented soybean paste, which is ripened at NaCl concentrations of >12% (w/w). We hypothesized that *B. licheniformis* would be a potential doenjang starter candidate among the identified *Bacillus* species from doenjang based on its appearance frequency and salt tolerance on tryptic soy agar (TSA) supplemented with 14% (w/v) NaCl [1].

Recently, Dunlap *et al*. separated *Bacillus paralicheniformis* from *B. licheniformis* on the basis of phylogenomic and phylogenetic studies [2]. They reported that urease activity is a phenotypic property that can differentiate between the two species. Urease activity, which converts urea into ammonia and carbamate, has been found in several bacteria, as well as fungi, plants, and some invertebrates. It has also been shown to be an important virulence factor in several pathogenic bacteria [3, 4]. The existence of urease is well known in *Helicobacter* spp., including all *Helicobacter pylori* isolated from gastritis patients [3, 4]. About 2% of *Clostridium perfringens* strains, an etiologic factor of gas gangrene, were reported to harbor urease genes localized on plasmids [5], and a strain of *Vibrio parahaemolyticus*, a species generally considered non-ureolytic which also produces urease, has been isolated [6]. Numbers of studies on the pathologic effect of bacterial ureases in human diseases have been performed, and urease involvement in several diseases, including urinary stones occurrence, catheter blockage, pyelonephritis, ammonia encephalopathy, hepatic coma, and gastritis has been reported [4].

The possession of a potential virulence factor is driving the need to clearly identify *B. paralicheniformis* from closely related *Bacillus* species. Because *B. paralicheniformis'* closest relative, *B. licheniformis*, is often purposely introduced in many bioindustries, including food industries, we

reexamined the validity of using urease as a *B. paralicheniformis* taxonomic index, and developed a new taxonomic marker that can discriminate the species from its nearest phylogenetic relatives.

Materials and Methods

Bacillus Strains and Cultures

Ninety strains isolated from Korean soybean-based fermented foods, and identified as *B. licheniformis* were subjected to multilocus sequence typing (MLST) and urease characterization. The isolate sources follow: meju, a spontaneously fermented and dried soybean brick; doenjang, a fermented soybean paste; ganjang, a soy sauce made from meju; gochujang, a fermented red pepper paste made with meju; and cheonggukjang, a soybean food fermented with *Bacillus* species (Table 1). The soybean-based fermented foods were collected from several districts in Korea. Among the 90 strains, 62 strains were isolated, identified, and stored in our stock cultures [7, 8], 23 strains were kindly provided by the Microbial Institute for Fermentation Industry (http://mifi.kr), and five strains were kindly provided by the Korea Food Research Institute (http://www.kfri.re.kr). Reference strains, *B. licheniformis* DSM 13^T (KACC 10476^T), *B. licheniformis* ATCC 9789 (KCTC 1659), *B. paralicheniformis* KJ-16^T (KACC 18426^T), *B. paralicheniformis* NCIMB 8874 (KCTC 3056), and *B. sonorensis*

Table 1. Polymorphism of eight housekeeping genes in the strains used in this study and corresponding sequence type (ST), frequency, and urease activity.

ST	Allelic profile								Frequency	Geographical origin	Sample	Urease activity	Reference strain
	adk	*ccpA*	*glpF*	*gmk*	*ilvD*	*pur*	*spo0A*	*tpi*					
1	1	1	1	1	1	1	1	1	1	Jeolla (1)	Cheonggukjang (1)	–	
2	1	1	1	2	1	1	1	1	1	UK (1)	NA (1)	–	DSM 13^T
3	2	1	1	1	1	1	1	1	2	Gyeonggi (1), Jeolla (1)	Doenjang (1), Ganjang (1)	–	14ADL4
4	2	1	1	1	2	1	1	1	30	Jeolla (30)	Doenjang (30)	–	
5	2	1	1	1	2	2	1	1	2	Jeolla (2)	Doenjang (2)	–	
6	2	1	1	1	3	1	1	1	9	Gangwon (3), Jeolla (5), Seoul (1)	Cheonggukjang (2), Doenjang (2), Gochujang (2), Meju (3)	–	
7	2	1	1	1	3	1	2	1	2	Jeolla (2)	Cheonggukjang (1), Gochujang (1)	–	
8	2	1	1	1	3	1	3	1	1	Seoul (1)	Meju (1)	–	
9	2	1	1	3	2	2	1	1	1	Jeolla (1)	Doenjang (1)	–	
10	2	1	1	3	3	1	1	1	3	Gangwon (1), Jeolla (2)	Cheonggukjang (1), Doenjang (1), Gochujang (1)	–	
11	2	1	2	1	2	1	1	1	1	Jeolla (1)	Doenjang (1)	–	
12	2	2	1	1	1	1	4	1	1	UK (1)	Milk (1)	–	ATCC 9789
13	2	3	1	1	1	1	1	1	4	Gangwon (2), Jeolla (2)	Cheonggukjang (1), Doenjang (1), Gochujang (1), Ganjang (1)	–	
14	2	3	2	1	1	1	1	1	1	Gangwon (1)	Doenjang (1)	–	
15	2	4	1	1	1	1	1	1	1	Jeolla (1)	Doenjang (1)	–	
16	3	1	3	4	4	3	5	2	1	Seoul (1)	Meju (1)	–	
17	3	1	4	5	5	4	5	2	1	NA (1)	Septic wound (1)	+	NCIMB 8874
18	3	2	3	4	4	3	5	2	21	Gyeonggi (20), Jeolla (1)	Doenjang (14), Meju (7)	–	14DA11
19	3	2	3	4	4	3	6	2	1	Gyeonggi (1)	Doenjang (1)	–	
20	3	2	3	4	4	5	5	2	3	Gyeonggi (3)	Doenjang (1), Meju (2)	–	
21	3	2	3	4	6	3	5	2	1	Gyeonggi (1)	Meju (1)	–	
22	3	2	3	6	7	6	7	2	1	Chungbuk (1)	Cheonggukjang (1)	+	KJ-16^T
23	3	5	3	5	4	2	5	3	6	Gangwon (3), Gyeonggi (3)	Doenjang (5), Ganjang (1)	+	
24	3	5	5	5	8	7	5	2	1	Gangwon (1)	Doenjang (1)	–	

Jeolla, Gangwon, Seoul, Gyeonggi, and Chungbuk are administrative districts in South Korea. The numbers in parentheses indicate the number of isolates. NA: not available.

NBRC 101234^T (KCTC 13918^T) were purchased from the Korean Agricultural Culture Collection (http://genebank.rda.go.kr/) and the Korean Collection for Type Cultures (http://kctc.kribb.re.kr/). Reference strains *B. licheniformis* 14ADL4 and *B. paralicheniformis* 14DA11 were isolated from doenjang; the complete genome sequences have been published [9, 10]. *B. sonorensis* strain SRCM101395 was kindly provided by the Microbial Institute for Fermentation Industry. All *Bacillus* isolates were cultured in TSA (Difco, USA) and tryptic soy broth (TSB; Difco) at 30°C for 24 h.

Urease Activity Test

The strain phenotypic urease activities were tested using Christensen's urea agar (KisanBio, Korea). The agar plates were prepared according to the manufacturer's instructions. Colonies cultured on TSA were inoculated onto urea agar plates and incubated at 37°C for 24 h, and color changes from yellow to purple-red were monitored.

Target Gene Amplification and Sequencing

We performed MLST using the following eight housekeeping genes: *adk* (encoding adenylate kinase), *ccpA* (catabolite control protein A), *glpF* (glycerol facilitator), *gmk* (guanylate kinase), *ilvD* (dihydroxyacid dehydratase), *pur* (phosphoribosyl aminoimidazole carboxamide formyltransferase), *spo0A* (stage 0 sporulation protein A), and *tpi* (triosephosphate isomerase). The target genes *adk*, *ccpA*, and *spo0A* were selected from a previously published *B. licheniformis* MLST scheme [11]; the others were selected from a *B. cereus* group study [12]. *B. licheniformis* DSM 13^T (GenBank accession number: GCA_000011645.1), *B. licheniformis* 14ADL4 (GCA_002966955.1), *B. paralicheniformis* KJ-16^T (GCA_001042485.2), and *B. paralicheniformis* 14DA11 (GCA_002393225.1) genome sequences were used to design PCR primers to amplify the internal regions of the eight genes, generating fragments of 475–627 bp (Table 2).

Genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen, Germany) after cell wall lysis with 1 mg/l lysozyme (Sigma, USA) at 37°C for 1 h. Amplification of the housekeeping genes for MLST was performed using specific primer sets (Table 2) and a T3000 Thermocycler (Biometra, Germany). Samples were preheated at 95°C for 5 min, and then amplified by 35 cycles of 95°C for 30 sec, 63°C for 45 sec, and 72°C for 60 sec. Reactions included template DNA (10 ng), 1 U of *Taq* polymerase (InClone, Korea), 0.5 μM each primer, 10 mM dNTPs, and 2.5 mM $MgCl_2$. The PCR products were purified using a PCR purification kit (SolGent, Korea), and sequenced by GenoTech (Korea) with the specific primer sets used for their original amplification. Sequence similarities were identified using the BLASTX program at the National Center for Biotechnology Information (NCBI) website. All sequence data produced in this study have been deposited in the NCBI database under accession numbers MH349499–MH349537.

Multilocus Sequence Typing and Phylogenetic Analysis

The sequences of the eight genes were edited, and the complementary sense and antisense fragments were aligned using LaserGene 7.1 software (DNASTAR, USA). The gene sequences were manually concatenated in the order *adk*, *ccpA*, *glpF*, *gmk*, *ilvD*, *pur*, *spo0A*, *tpi*, and then aligned using the multiple sequence

Table 2. Genes and PCR primers used for the MLST of 90 strains formerly identified as *B. licheniformis*.

Gene	Target gene	Forward (5′–3′)	Reverse (5′–3′)	Amplicon size (bp)	Analyzed sequence size (bp)	No. of alleles	No. of polymorphic sites	Reference
adk	Adenylate kinase	GGTAAAGGGAC ACAGGCTGA	TCGAGTAAAGG CTGGGTTTG	518	438	3	6	[11]
ccpA	Catabolite control protein A	TATGATGTAGCA CGCGAAGC	TATCCCCAAGCG CTCTTTTA	604	517	5	24	[11]
glpF	Glycerol facilitator	CGGAACGATGCT GCTCATCG	CAATCGGAAGG ACAAAATGGGC	578	505	5	21	This study
gmk	Guanylate kinase	CGTTCTCTCCGG CCCTTCCG	CGGCGAGAACG ATTGCTTTG	533	415	6	15	This study
ilvD	Dihydroxyacid dehydratase	GACTGTAACGGG GAAAACGC	GCCGGGCCAGTT TGCTTTTC	563	497	8	22	This study
pur	Phosphoribosyl aminoimidazole carboxamide formyltransferase	GCGCTGCAGCG AAAAACCAC	GCGGTTCAGGGC GATGATGC	536	477	7	29	This study
spo0A	Stage 0 sporulation protein A	GAAGTGCTTGGT GTCGCATA	TGTGTAGCCGAA AAGTGACG	627	541	7	30	[11]
tpi	Triosephosphate isomerase	GCCCTGCGGCTT TGAAAGAC	CTTGCTCCGCCA ACAAGAGC	475	413	3	8	This study

alignment program ClustalW [13]. Phylogenetic analysis of the aligned sequences was performed using the maximum likelihood method in MEGA 7 [14]. Bootstrapping values are estimated from 1,000 repeated calculations. Reference strain gene sequences were obtained from NCBI. Allele sequences from each gene were designated allele numbers, and used to generate sequence types (STs) for each isolate.

Results

Phylogenetic Position of *B. paralicheniformis* Based on the 16S rRNA Gene

Our phylogenetic analysis of *B. paralicheniformis* strains near complete 16S rRNA gene sequences, together with those from other *Bacillus* species/strains, revealed that *B. paralicheniformis* is closely related not only to *B. licheniformis*, but also to *B. sonorensis* (Fig. 1). The type strains of the three species shared >99.5% 16S rRNA gene sequence identity.

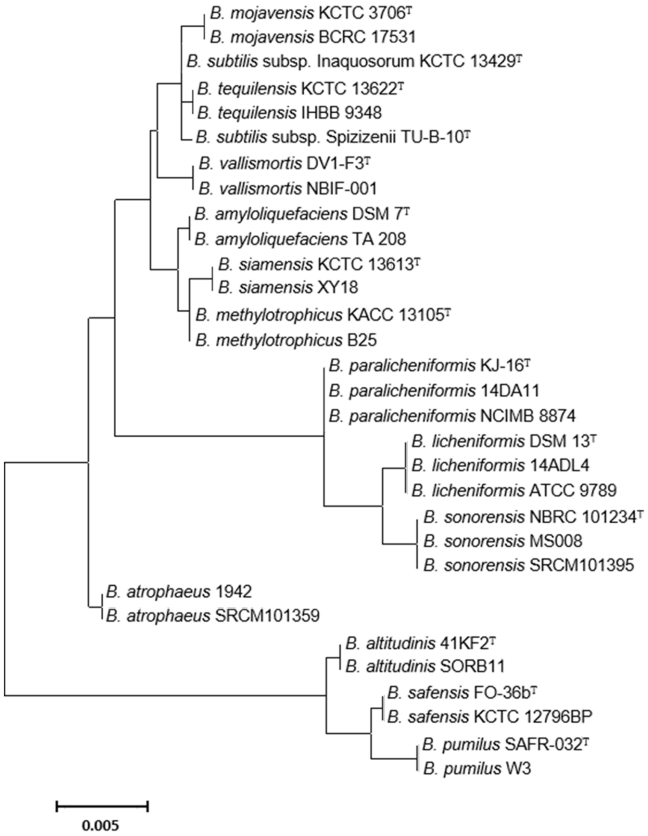

Fig. 1. Maximum likelihood phylogenetic tree based on entire 16S rRNA gene sequences from *Bacillus paralicheniformis* and its relatives.

Branches with bootstrap values lower than 50% were collapsed. A nucleotide/site distance scale is shown under the tree.

We identified five polymorphic sites, positions 67, 70, 86, 88, and 280, on the 16S rRNA gene sequence that can differentiate *B. paralicheniformis* from *B. licheniformis* and *B. sonorensis* (Table S1). However, the nearly identical 16S rRNA gene sequences shared among the three species necessitates the development of additional phylogenetic markers to differentiate between the species unequivocally.

Urease Gene Clusters in *B. paralicheniformis* and Its Relatives

To confirm that urease activity can be used as a phenotypic property to differentiate *B. paralicheniformis* from *B. licheniformis* and *B. sonorensis*, we tested the urease activity of reference strains of the three species, in the same manner as per Dunlap *et al*. [2]. Unlike the Dunlap *et al*. study, tested *B. paralicheniformis* strains exhibited varying levels of urease activity, in a strain-specific manner, down to none, in our study. However, as in the previous report's species-specific manner, none of the *B. licheniformis* or *B. sonorensis* strains exhibited the activity (Fig. S1). Among the 90 strains tested in our study, eight strains exhibited phenotypic urease activity (Table 1).

To shed light on the genetic background behind the strain-specific urease activity of *B. paralicheniformis*, we analyzed urease genomic data for *B. paralicheniformis*, *B. licheniformis*, and *B. sonorensis* obtained from NCBI. As of our analysis (July 7, 2018), the genome sequences of 76, 17, and five strains of *B. licheniformis*, *B. paralicheniformis*, and *B. sonorensis*, respectively, had been deposited at NCBI. Among the 76 *B. licheniformis* strains, ten strains harbor the urease gene cluster *ureABCEFGDH*, and two strains possess the gene cluster *ureABC*; however, the remainder do not have a urease gene cluster. Notably, among the 12 strains having a urease gene cluster, the taxonomic position of nine is questionable based on 16S rRNA gene polymorphic sites and housekeeping genes (Table 3). Among the 17 *B. paralicheniformis* strains, all possess *ureABCEFGDH*, except strain NMSW12 (GCA_002998255.1), which does not possess a urease gene. Considering that the strain NMSW12 published genome is incomplete, we cannot rule out the possibility of the strain possessing the gene. Among the five *B. sonorensis* strains, four harbor the urease gene cluster *ureABC*, and one has *ureABCEFGDH* (Fig. S2). These results demonstrate that most *B. licheniformis* strains do not harbor a urease gene cluster, and that the general urease gene cluster types of *B. paralicheniformis* and *B. sonorensis* are *ureABCEFGDH* and *ureABC*, respectively. However, unusual cases can be found within each species.

Table 3. Strains harboring a type of urease gene cluster identified from 76 *B. licheniformis* strains with published genomes.

Strain	Urease type	Sequence type									Identification
		16S rRNA	*adk*	*ccpA*	*glpF*	*gmk*	*ilvD*	*pur*	*spo0A*	*tpi*	
LMG 7559	*ureABCEFGDH*	P	P	P/L	P	P	P	NS	P	P	*B. paralicheniformis*
LMG 6934	*ureABCEFGDH*	P	P	NS	NS	P	P	NS	P	P	*B. paralicheniformis*
YNP2-TSU	*ureABCEFGDH*	L	L	L	L	L	NS	NS	L	L	*B. licheniformis*
YNP3-TSU	*ureABCEFGDH*	L	L	L	L	L	NS	NS	L	L	*B. licheniformis*
B4121	*ureABCEFGDH*	P	P	P/L	NS	P	NS	NS	NS	P	*B. paralicheniformis*
7510	*ureABCEFGDH*	P	P	NS	P/L	P	P	P	P	P	*B. paralicheniformis*
B4123	*ureABCEFGDH*	P	P	P/L	NS	P	P	NS	P	P	*B. paralicheniformis*
B4125	*ureABCEFGDH*	P	P	P/L	P/L	P	P	P	P	P	*B. paralicheniformis*
S 16	*ureABCEFGDH*	L	NS	NS	NS	NS	NS	NS	NS	NS	*B. licheniformis*
S27	*ureABCEFGDH*	P	P	P/L	P/L	P	P	P	P	NS	*B. paralicheniformis*
SRCM101441	*ureABC*	S	NS	NS	NS	NS	NS	NS	NS	NS	*B. subtilis*
127185/2	*ureABC*	S	NS	NS	NS	NS	NS	NS	NS	NS	*B. subtilis*

Genetic information was retrieved from the NCBI database on July 7, 2018.

Abbreviations: P, *B. paralicheniformis* sequence type; L, *B. licheniformis* sequence type; P/L, a common sequence type possessed by *B. licheniformis* and *B. paralicheniformis*; S, *B. subtilis* sequence type; NS, not identified sequence type in this study.

Discrimination of *B. licheniformis* and *B. paralicheniformis* Based on MLST

MLST is a highly discriminative typing method appropriate for genetically coherent organisms that can provide a measure of genetic relatedness among strains [15]. MLST data, as well as its constituent housekeeping gene sequences, have often been used to distinguish between species exhibiting close phylogenetic relatedness.

We used eight MLST housekeeping genes to discriminate between *B. licheniformis* and *B. paralicheniformis* (Table 2), and applied the scheme to 90 isolates formerly identified as *B. licheniformis*. Our analysis was based on eight phylogenomically defined reference strains: *B. licheniformis* (DSM 13^T, 14ADL4, and ATCC 9789), *B. paralicheniformis* (KJ-16^T, 14DA11, and NCIMB 8874), and *B. sonorensis* (NBRC 101234^T and SRCM101395). The number of different variants for each gene ranged from three to eight, but each variant gene had >95% identity to its counterpart in *B. paralicheniformis* KJ-16^T (Tables 1 and 2). The most variable locus, *spo0A*, had 30 polymorphic sites, and *adk* was the least variable, with only six polymorphic sites. We generated a maximum likelihood phylogenetic tree based on a concatenated alignment of the eight gene sequences for the 90 strains. The tree divides into two primary groups, with each group including appropriate *B. licheniformis* and *B. paralicheniformis* reference strains (Fig. 2). Two *B. sonorensis* reference strains cluster in a separate branch of the tree. Among the 90 strains we subjected to MLST, 33 strains were reidentified as *B. paralicheniformis*.

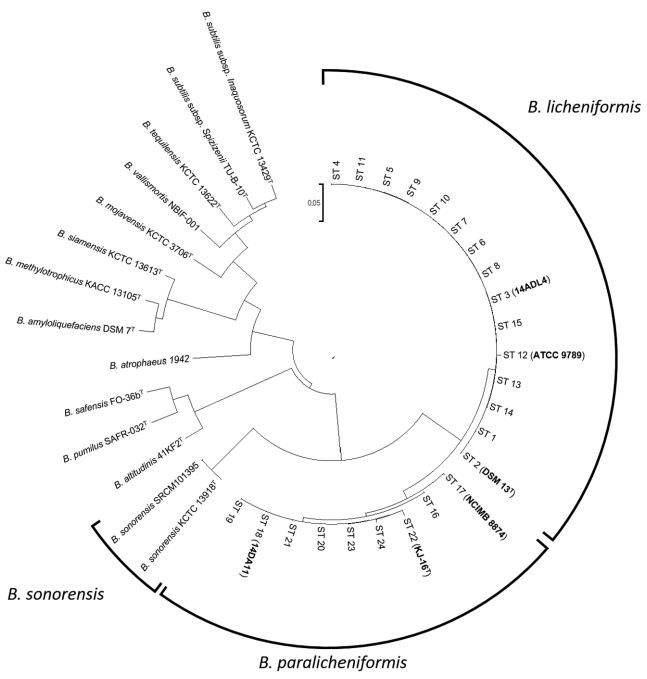

Fig. 2. Maximum likelihood phylogenetic tree based on the sequence types of 90 strains formerly identified as *B. licheniformis*.

The reference strains of this study are indicated in parentheses.

spo0A, an Auxiliary Marker for *B. paralicheniformis* Identification

Although MLST clearly discriminates between

B. licheniformis, *B. paralicheniformis*, and *B. sonorensis*, it is not a simple and rapid method, compared with qualitative PCR methods. Among the eight housekeeping genes, *adk* and *tpi* have fewer polymorphic sites than the others. Furthermore, some *ccpA* and *glpF* sequence types are commonly shared by *B. licheniformis* and *B. paralicheniformis*. Therefore, these four genes are not sufficiently informative to discriminate between the species. However, we identified a sufficient number of polymorphic sites in the *spo0A* sequence to discriminate between the three species. We, therefore, constructed a *spo0A*-specific PCR primer set for the rapid identification of *B. paralicheniformis* (Forward, 5-CATCGCGATGAATTCTGA-3; Reverse: 5-CATGGA GAACTTAGTCGGC-3). The primer set perfectly separated *B. paralicheniformis* from *B. licheniformis* and *B. sonorensis* (Fig. S3), which demonstrates the potential utility of *spo0A* as a molecular marker for the rapid identification of bacilli. Analysis of 16S rRNA and *spo0A* gene sequences together allow for the accurate and rapid identification of *B. licheniformis*, *B. paralicheniformis*, and *B. sonorensis*, apart from other bacilli. A *spo0A*-specific PCR amplification against the 90 strains tested in our study further corroborates our MLST results (data not shown).

Discussion

The precise discrimination of similar bacterial species, and a concurrent increase of novel species identification, have been made possible by rapid progress in molecular taxonomic methodology. The introduction of phylogenomic approaches has enhanced the accuracy of bacterial taxonomic studies and clarified the consolidation and separation of closely related species [2, 16, 17]. The precise identification of *B. paralicheniformis* is more relevant than merely determining taxonomic position: most of the species' strains possess a urease gene cluster, which is considered a pathogen virulence factor. Furthermore, its closest relative, *B. licheniformis*, is actively used in many bioindustries. *B. licheniformis* can be intentionally added to foods or feeds in the European Union, because the European Food Safety Authority qualifies the species as a safe biological agent [18], and the US Food and Drug Administration allows the species to be used for genetically modified organism enzyme production.

We found urease activity and urease gene cluster possession not to be a species-specific property between *B. paralicheniformis* and *B. licheniformis*, as the latter harbors a urease gene cluster, albeit rarely. The *B. paralicheniformis* urease gene cluster may not be involved in human disease, and rather in nitrogen utilization, considering its habitat. The majorities of *Bacillus* spp., including *B. paralicheniformis*, have been isolated from soil and the rhizosphere [19, 20]. However, we cannot speculate on the results were this species to be used for human or animal food production, because no report of its safety aspects has yet been published. Therefore, prudency dictates that *B. paralicheniformis* be excluded from human and animal use, until further study.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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